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Modulation of Lymphocyte Locomotion through Type I Collagen by Anti-β_1 Integrin Antibodies

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Abstract

We investigated the role of collagen-binding VLA integrins in lymphocyte locomotion using selected antibodies specific to distinct β_1 epitopes and two (different) α chains in an attempt to disrupt lymphocyte interaction with the collagen matrix and thereby alter the net movement of lymphocytes through the matrix. Results indicate that antibody 33B6 to the β_1 1.2 epitope, when simultaneously introduced to the cells with collagen substrate, could inhibit lymphocyte locomotion of both PBMC and donor-matched anti-CD3 / IL-2 activated T cells (aT) into type I collagen. Antibody TS2-7 to the α_1 chain of VLA-1 stimulated locomotion of both PBMC and aT. We hypothesize that these antibodies modulate the interaction of integrin receptor with collagen substrate thus altering locomotory behavior through collagen *in vitro*.

Introduction

Immunity *in vivo* is critically dependent on the ability of lymphocytes to locomote through extracellular matrix (ECM). Locomotion through interstitium is central to both tissue inflammation and lymphocyte trafficking through lymphoid tissue. Little is known about how lymphocytes and ECM interact to initiate and sustain locomotion. Cell receptor-ECM ligand interactions have been observed to influence cell behavior (1,2,3) and may influence the process of lymphocyte locomotion. To facilitate understanding of the complex interaction between cells and ECM, we investigated single component interactions with specific cell-surface receptors within an *in vitro* model matrix.

Research in lymphocyte locomotion currently uses three-dimensional, gelled type I collagen, a major ECM component of the interstitium (19 -21) as the basic model for lymphocyte movement through interstitium. Lymphocytes interact with components of the ECM via an array of cell surface-expressed adhesion molecules. One family of adhesion molecules, the Integrins, bind to collagen. The molecules VLA-1, -2, and -3 of the very late activation (VLA) antigen integrin subfamily have specificity for type I collagen (9). These VLA molecules are composed of two transmembrane chains; a unique α chain which determines binding specificity and a common ß chain ($ß_1$). The interaction of the $ß_1$ chain with a specific antibody or ligand may influence cell behavior (4,5,6). Adhesion molecule binding to collagen is highly redundant, therefore we used antibodies to $ß_1$ chain to address

the role of several collagen-binding VLAs in locomotion. In addition, antibodies specific to the α_1 and α_2 chains of two specific collagen binding $ß_1$ integrins, VLA-1 and VLA-2, respectively, were used.

We investigated the effect of these specific anti-VLA antibodies on lymphocyte locomotion via incorporation into a modified locomotion microassay (7). Results suggest that the $ß_1$ molecule may be essential to proper lymphocyte interaction with collagen as the cell locomotes through the ECM of the interstitium.

Materials and Methods

Cells

Peripheral blood mononuclar cells (PBMC) were isolated by Ficoll-Hypaque gradient (Pharmacia Biotech, Piscataway, NJ) from human buffy coats obtained from Gulf Coast Regional Blood Center (Houston, Tx). Activated cells were obtained by incubating isolated PBMC with 1:1000 dilution anti-CD3 ascites fluid (provided by Dr. Bradley W. McIntyre, University of Texas M.D. Anderson Cancer Center, Houston, Tx) and 100 U/ml human recombinant interleukin-2 (IL-2; Genzyme, Cambridge, MA.) for at least seven days prior to use in assay. Cultures were maintained in complete media prepared as RPMI-1640 medium supplemented with 5% penicillin-streptomycin (Gibco, Grand Island, NY) and 10% Fetal Bovine Serum (Hy-Clone Laboratories, Logan, UT).

Antibodies

Mouse anti-human VLA monoclonal antibodies (MAb) 33B6 and 18D3 (anti-$ß_1$, 1.2 and 1.1 epitopes, respectively) and TS2-7 (anti-α_1) were derived from mouse ascites fluid provided by Dr.Bradley McIntyre (University of Texas M.D. Anderson Cancer Center, Houston, Texas). The MAb A1A5 (anti-ß1, 1.3 epitope) was purchased from T Cell Diagnostics (Cambridge, MA). The MAb P1E6 (anti-α_2) was obtained from Telios (San Diego, CA).

Locomotion Assay

Lymphocyte locomotion was assessed *in vitro* by measuring cell movement through a three-dimensional matrix of type I collagen in a modification of previously described assays (7,8). Collagen was extracted from rat tail tendons (Pel-Freeze, Rogers, AK) as described by Ratner et al. (1988). Stock preparations of 1.2 - 1.8 mg collagen per ml of 0.1% glacial acetic acid were maintained at 4°C for up to a year. These stock solutions of 100% collagen were diluted with 0.1% glacial acetic acid to 80% (0.96 - 1.44 mg/ml) or 90% (1.08 - 1.30 mg/ml) collagen as needed.

Collagen matrices were prepared by adding concentrated RPMI-1640, 7.5% $NaHCO_3$, 0.34M NaOH to diluted collagen on ice to inhibit polymerization. Antibody-treated collagen matrices were prepared by diluting each antibody 1:100 in 100 µl aliquots of the unpolymerized collagen prior to distribution in 10 µl aliquots into Terasaki microtest tray wells. Both untreated and antibody-treated collagen were then allowed to polymerize at 37°C in 5% CO_2 for 10 min. Cells (2×10^5) were suspended in 100 µl complete RPMI-1640 medium alone for the control samples or with 1:100 dilution of antibody for the test samples. Cells were then, dependent on the experiment, either preincubated on ice or directly overlaid in 15 µl aliquots on seperate wells of polymerized collagen matrix. The microtest trays were incubated at 37°C in 5% CO_2 for more than 10 - 20 hours.

Lymphocyte locomotion was defined as the distance from the cell overlay-matrix interface, inward through the collagen, to a point within the matrix where only three cells could be focused in one field at 40X magnification. Distances were measured in fine-focus units on an inverted light microsope (Nikon, Diaphot, Japan) and then converted to micrometers (μm). All tests were performed in sextuplicate. Locomotion distances are depicted as the mean μm ± standard deviation. Significant differences in locomotion distance relative to controls were determined by ANOVA with Statview 512+ (Abacus Concepts, Calabasas, CA).

Results

Only two of the anti-VLA antibodies investigated showed a significant effect on lymphocyte locomotion after 10 hours incubation (Fig 1a). Monoclonal antibody TS2-7 against the α_1 chain of the collagen-binding integrin VLA-1 stimulated locomotion. Cells treated with TS2-7 travelled further into the collagen matrix (279.75 ± 9.51 μm) at a distance that was statistically greater than did untreated cells (200.83 ±7.93 μm) ($p < 0.05$). This effect persisted after 20 hours (TS2-7, 436.63 ± 18.52 μm; media control, 337.71 ± 38.13 μm). The MAb P1E6, against the α_2 chain of another collagen-binding integrin, VLA-2, had no significant effect on locomotion after either 10 or 20 hours.

The MAb 33B6 (against the ß1.2 epitope of the VLA $ß_1$ chain) stimulated locomotion (254.69 ± 28.11 μm) above that seen in untreated cells (200.83 ± 7.93 μm). This effect persisted after 20 hours (33B6, 384.53 ± 24.55 μm; control, 337.71 ± 38.13 μm). Neither MAb A1A5 (against the putative ß1.3 epitope) nor 18D3 (against the ß1.1 epitope) significantly affected locomotion after either 10 or 20 hours.

The PBMC from this same donor were activated for at least seven days with anti-CD3 ascites fluid and recombinant human IL-2. Following activation, the locomotion assay was repeated as previously performed for unactivated PBMC locomotion (Fig 1b). The MAb 33B6 significantly inhibited locomotion (324.69 ± 32.44 μm) relative to untreated cells (490.52 ± 14.27 μm). This effect persisted after 20 hours (33B6, 595.63 ± 32.36 μm; control, 729.17 ± 8.19 μm). None of the other

antibodies, including TS2-7, affected the locomotion of activated lymphocytes after either 10 or 20 hours incubation.

Omission of preincubation with antibody (Fig 2) changes the response of the cells to the introduction of matrix. Fresh PBMC from a seperate donor were suspended in either media alone or antibody-treated media and overlaid directly on the surface of antibody-treated 80% collagen matrix. As shown in Fig 2a, after 10 hours, cells treated with either of the α chain antibodies TS2-7 and P1E6 (418.33 ± 30.20 and 382.08 ± 18.58 μm, respectively) migrated further into the collagen matrix than the untreated control cells (381.25 ± 21.34 μm). This effect persisted after 20 hours (TS2-7, 560.00 ± 9.01 μm; P1E6, 525.31 ± 20.29 μm; control, 471.25 ± 25.04 μm). Two $ß_1$ antibodies, 33B6 and 18D3 were slightly inhibitory. No significant difference in locomotion distance was seen in cells treated with the other antibodies (Fig 2a).

Locomotion of activated, donor-matched lymphocytes was altered in response to simultaneous contact with antibody and collagen with only two of the antibodies tested (Fig 2b). After 10 hours, the presence of 33B6 abtibody inhibited movement into the matrix by approximately 21% of the control distance. Cells traveled 507.81 ± 14.69 μm into the 33B6-treated matrix, whereas the untreated control traveled 643.85 ± 5.76 μm. This inhibotory effect persisted after 20 hours (33B6, 604.69 ± 12.76 μm; control, 742.81 ± 7.02 μm). After 10 hours, TS2-7-treated cells traveled 726.46 ± 5.64 μm into the matrix, a significant increase ($p<0.05$) over the untreated control (643.85 ± 5.76 μm). This modest stimulatory effect persisted after 20 hours.

Cells from another donor were also tested for migration in antibody-treated collagen matrices (Fig 2c). Locomotion of PBMC after 10 hours (Fig 2c) was effected by all but one of the antibodies tested (18D3). Both α chain antibodies stimulated locomotion; TS2-7 and P1E6 MAbs stimulated cells to penetrate further into the collagen than the untreated control cells (TS2-7, 276.25 ± 15.10 μm; P1E6, 287.50 ± 10.36 μm; control, 232.60 ± 15.41 μm). The three $ß_1$ antibodies had more varied effects. Treatment with MAb A1A5 seemed to stimulate locomotion but the distances traveled were not statistically different from control ($p<0.05$). The MAb 33B6 significantly inhibited cell movement into the collagen (181.46 ± 8.19 μm). Only MAb 18D3 did not affect mean locomotion distance.

Donor-matched lymphocytes activated as previously described were affected by treatment with only two of the antibodies investigated (Fig 2d). After 10 hours, TS2-7-treated cells migrated 618.23 ± 11.84 μm, versus only 549.79 ± 7.03 μm for the control. Locomotory distance in the presence of MAb 33B6 at 457.92 ± 6.93 μm was statistically less than that in the untreated control (549.79 ± 7.03 μm).

Potential synergy between the inhibitory activity of anti-$ß_1$ antibodies was also investigated (Fig 3). Lymphocytes from another donor were activated as previously described. Solutions of 1:100 dilution of each antibody in complete media were used directly to resuspended activated cells. Cells treated with MAb 33B6 traveled a mean distance of 594.22 ± 11.79 μm into the collagen matrix; untreated control cells

traveled only 699.48 ± 10.97 μm. The MAb 18D3 also inhibited locomotion (613.07 ± 11.79 μm) to a slightly lesser extent than did 33B6. The presence of both anti-β_1 antibodies in the assay restored locomotion to the control level.

In summary, as depicted schematically in Fig 4, lymphocyte locomotion through collagen was altered in response to treatment with antibodies to different components of the collagen-binding antigens VLA-1 and VLA-2. The effects on locomotion differed with method of antibody treatment. Lymphocytes preincubated in the absence of collagen with MAb 33B6 consistently exhibited locomotion that was stimulated (Fig 4b) beyond that seen in untreated controls (Fig 4a). Identical treatment with MAbs 18D3 and A1A5 did not influence locomotion with either statistical significance or any real consistency (Fig 4b). Collagen-free preincubation with MAb TS2-7 also stimulated locomotion with consistency whereas MAb P1E6 showed neither consistent nor significant influence on locomotion (Fig 4c). Simultaneous contact of lymphocytes with both MAb 33B6 and type I collagen resulted in significant inhibition of locomotion (Fig4d).

Discussion

Immune surveillance *in vivo* is dependent on the ability of lymphocytes to locomote through ECM. The activity of cellular adhesion to, and subsequent release of, ECM components such as collagen is central to the process of lymphocyte locomotion. Lymphocytes interact with ECM components through surface-expressed dimeric adhesion molecules known as integrins, specifically, the subfamily of integrins that share a common beta chain ($ß_1$) and are known as very late activation antigens (VLA) (9). The role of these $ß_1$ integrins in lymphocyte locomotion was investigated using antibodies specific to distinct VLA chain determinants in an attempt to disrupt lymphocyte interaction with the type I collagen matrix and thereby alter the net movement of lymphocytes through the assay matrix.

Antibodies to two different type I collagen-binding VLA $ß_1$ integrins had pronounced effects on lymphocyte locomotion *in vitro*. In the presence of type I collagen, the anti-$ß_1$ Mab 33B6 consistently inhibited the locomotion of anti-CD3/IL-2 activated lymphocytes, and less consistently of donor-matched PBMC (Figs 1b and 2a-d). Pretreatment with 33B6 MAb prior to contact with type I collagen persistently stimulated locomotion in unactivated PBMC but not in donor-matched activated lymphocytes (Fig 1a). The anti-α_1 MAb TS2-7 consistently stimulated the locomotion of both PBMC and donor-matched activated lymphocytes (Figs 1a and 2a-d). Together, these data suggest a role for the collagen binding $ß_1$ integrins in regulating lymphocyte locomotion. We conclude that antibodies to selected VLA

determinants modulate the interaction of integrin receptor and collagen substrate resulting in an alteration of locomotory behavior through type I collagen.

Antibodies 33B6 and 18D3 bind the ß1.2 and ß1.1 epitopes of the $ß_1$ chain, respectively, and block cell adhesion to ECM substrate with different efficiencies (10). Cells are thought to propel themselves through substrate by exerting physical force against molecular contacts with collagen (11). Lymphocyte locomotion theoretically could be inhibited by physical blockage of collagen binding sites on the cells by antibody. Simultaneous treatment of cells with both 33B6 and 18D3 antibodies resulted in a level of locomotion statistically indistinguishable from that seen in control (Fig 3). This effect was not due to inactivity of the ascites used, because individual antibodies inhibited locomotion. Thus, the ability to block adhesion sites is not sufficient to consistently inhibit locomotion.

Important cellular functions such as ECM binding are highly duplicated systems. All three of the ß1 integrins that bind type I collagen (9) are elevated in T cells that migrate on type I collagen (12). The existence of both these multiple VLA collagen receptors as well as other, non-integrin collagen-binding molecules such as CD26 may be used by the cell to compensate for the physical blockage of particular $ß_1$ epitopes. A more likely explanation for the observed inhibtion with 33B6 antibody, however, is associated with the role of the antibody in stimulating homotypic aggregation between cells, which has been identified as the preliminary step in lymphocyte locomotion (13).

Three populations of cells arise in a locomotion assay: nonadherent, surface-adherent / nonmotile, and motile. Only 35% of an activated population of lymphocytes and less than 5% of unstimulated population of lymphocytes will actually become locomotory (14). Currently, the role of homotypic aggregation in the induction of locomotion is thought to facilitate the intercellular communication of information that initiates transformation into the locomotory phenotype (13). The communication may subsequently increase the affinity of cell surface receptors for ECM substrate thus facilitating the locomotion through collagen of cells that are less locomotory. Augmentation of locomotory potential has been observed upon activation of unstimulated PBMC (13,8).

Pretreating unstimulated PBMC with antibody 33B6 was found to stimulate locomotion (Fig 1a). This effect was not seen with donor-matched activated cells (Fig 1b) nor in the assays where pretreatment was omitted (Figs 2&3). This suggests that the binding of the β_1 chain by 33B6 in the absence of collagen may recruit cells into the locomotory potential. Antibody 33B6 stimulates homotypic aggregation in lymphocytes (10), and thus homotypic aggregation may function in increasing the locomotory potential of poorly locomotory cells. In the presence of activated lymphocytes, this "conversion" may cause these more locomotory cells to adhere too firmly to the collagen and thus impede locomotion (Fig 1b).

Similarly, the anti-β_1 antibodies 18D3 and A1A5 may interfere with the recruitment of cells into the locomotory phenotype by blocking homotypic aggregation (18). However, 18D3 abtibody inhibited locomotion within one assay (Fig 3) and A1A5 antibody stimulated locomotion, albeit to a lesser extent, (Figs 1a and 2c). Antibody that stimulates homotypic aggregation (33B6) has a definite effect on locomotion whereas antibody treatment that blocks homotypic aggregation (18D3) could be compensated for by other unidentified mechanisms.

In addition to homotypic aggregation, engaging the integrin receptor at β_1 could generate either a positive or negative signaling event. Recent findings that support this idea in human peripheral blood lymphocytes include an increase in binding affinity to ECM substrate in cells treated with anti-β_1 antibody (15) and the co-induction of T-cell proliferation by 33B6 itself (4).

Evidence also exists to support a negative signaling role for β_1 in lymphocyte activation (6,5,4). A negative signaling event for locomotion may be generated by engaging the β_1 chain at the ß1.2 epitope by 33B6. Introducing antibody at the same time as contact with collagen (Figs 2 & 3) inhibited locomotion. As antibody is bound to ß1, surrounding collagen matrix binds the adjoining α chain. With both chains bound simultaneously a specific conformational change occurs in the receptor that theoretically differs from that created by specific binding of antibody on the β_1 chain alone in the absence of collagen. Three-dimensional collagen substrate may place a physical constraint on the

receptor that positions the ß1.2 epitope in a conformation that favors sending a negative signal for locomotion when bound by antibody 33B6.

The creation of an epitope conformation by the physical positioning resulting from multiple binding events could also explain the behavior observed in Fig 4, in which lymphocytes were exposed simultaneously to a mixture of 33B6 and 18D3. Although 18D3 does not compete for binding with 33B6, it does block 33B6 effects (unpublished data). Binding of 18D3 may alter configuration of the receptor in a way that may both prevent binding of the ß1.2 epitope as well as negate the effect of previously bound ß1.2 epitope. Persistence of the inhibitory effect - as seen with the activated lymphocytes - (Figs 1 & 2) is consistent with the physical creation of an inhibitory configuration.

Our observation that α_1 and sometimes α_2 binding stimulated locomotion further supports the idea that subtrate binding creates a conformational change specific from that caused by anti-$ß_1$ antibody binding. Integrin α chains can differentially and specifically inducing cell behavior such as migration and collagen contraction in response to substrate-ligand binding (3,16). Although adhesion to substrate alone is insufficient to trigger migration (16), binding of α_1 may participate in the development of the asymmetricly distributed adhesion strengths that have been observed in migrating fibroblasts (17). The weaker membrane-only associated binding that was identified, which is dependent on the cytoplasmic domain of $ß_1$ chain, may differentiate from the strong, cytoskeletal associated binding also obsereved by some

conformational alteration of the β_1 chain by substrate-binding of an adjacent α chain. Therefore, anti-α_1 antibody TS2-7 and anti-$\alpha 2$ antibody P1E6 potentially mimic the adhesion molecule cross-linking that occurs in locomotion on substrate.

The conformational change created by three-dimensional substrate binding may therefore provide a way for cells to regulate their attachment to and release from substrate. A signal loop could be established from the substrate into the cell that allows modulation of cellular interactions such as adhesion, matrix contraction and locomotion back at the interface with the substrate. Modulation of these cellular interactions with substrate alters the three-dimensional configuration of the surrounding collagen matrix, altering conformational restraints, thus regulating the signals that continue this proposed signal loop.

In conclusion, the observation that antibody to the ß1.2 epitope of VLA β_1 chain inhibits movement into collagen suggests a role for the collagen-binding β_1 integrins in regulating lymphocyte locomotion. Engagement of integrin receptor by antibody in the presence or absence of substrate may result in signal events that differentially disrupt lymphocyte interaction with the collagen matrix, and thereby alter the net movement of lymphocytes through the matrix.

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Figure Legends

Figure 1 *Locomotion of lymphocytes preincubated with selected anti-VLA antibodies.* Isolated PBMC were suspended in complete RPMI-1640 containing 1:100 dilution antibody and assayed for locomotion on 90% rat tail type I collagen matrix. Cells tested were **a)** unstimulated PBMC from a human donor assayed after 10 hrs (open bar) and 20 hrs (hatched bar), and **b)** donor-matched, activated lymphocytes (cultured for at least 7-days with anti-CD3/IL-2) also assayed after 10 hrs (open bar) and after 20 hrs (hatched bar). Antibodies used: 18D3, 33B6 and A1A5 - anti-$\beta 1$ epitopes 1.1, 1.2, 1.3, respectively; P1E6 - anti-$\alpha 2$; and TS27 - anti-$\alpha 1$. (* = $p < 0.05$)

Figure 2 *Effect of simultaneous contact with selected anti-VLA antibodies and collagen substrate.* Cells were suspended in complete RPMI-1640 containing 1:100 dilution antibody and assayed on 80% rat tail type I collagen matrix that also contained 1:100 dilution antibody. Cells tested were **a)** unstimulated PBMC from a human donor assayed after 10 hrs (open bar) and after 20 hrs (hatched bar), **b)** donor-matched, anti-CD3/IL-2 activated lymphocytes (at least 7 days after activation) assayed after 10 hrs (open bar) and after 20 hrs (hatched bar), **c)** unstimulated PBMC from another human donor assayed after 10 hrs and **d)** donor-matched, anti-CD3/IL-2 activated lymphocytes (at least 7 days after activation) also assayed after 10 hrs. Antibodies used: 18D3, 33B6 and A1A5 - anti-β_1 epitopes 1.1, 1.2, 1.3 respectively; P1E6 - anti-α_2; and TS27 - anti-α_1. (* = $p < 0.05$)

Figure 3 *Cotreatment with two anti-β_1 antibodies.* Cells were suspended in complete RPMI-1640 treated with 1:100 dilution of two anti-β_1 MAbs and assayed on 80% rat tail type I collagen matrix. Cells tested were PBMC from a human donor activated at least 7 days with anti-CD3/IL-2, and then assayed for locomotory distance after 20 hrs. Antibodies used: 18D3 and 33B6 - anti-β_1 epitopes 1.1 and 1.2, respectively. (* = $p < 0.05$)

Figure 4 *Summary.* **a)** Cells suspended in complete media alone migrate a standard distance into model matrix. Standard distance traveled varied by donor and activation state of cells. These untreated samples, used as control for locomotion with antibody treatment, are not engaged by antibody at their integrin collagen type I receptor(s). **b)** Cell integrin receptor(s) bound by anti-β_1 antibody 33B6 in the absence of type I collagen resulted in stimulated locomotion. The anti-β_1 antibodies 18D3 and A1A5 had no significant effect on locomotion. **c)** Locomotion was consistently stimulated when integrin VLA-1 was engaged at the α chain by anti-α_1 antibody TS2-7. The anti-α_2 antibody P1E6, and thus integrin VLA-2, did not consistently effect locomotion with significance. **d)** Integrin bound simultaneously at the β chain by anti-β_1 antibody 33B6 and at the α chain(s) by type I collagen resulted in significantly inhibited locomotion.

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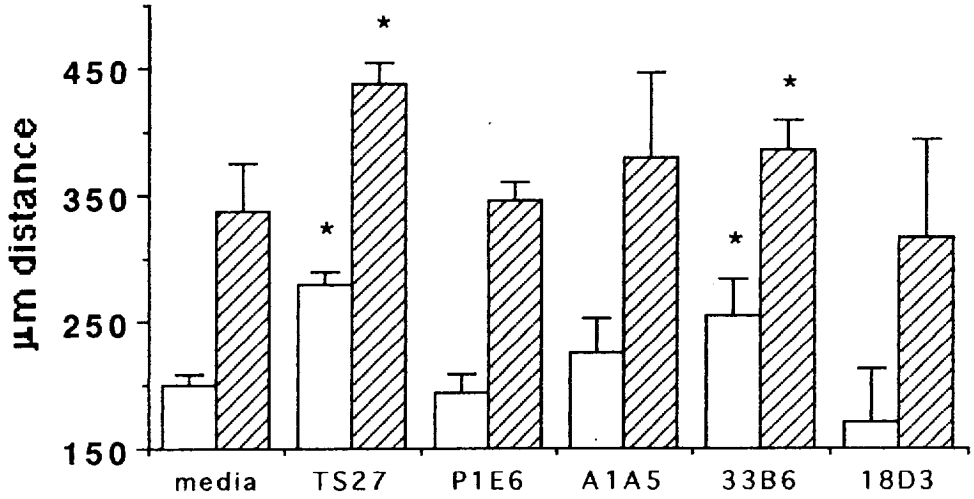
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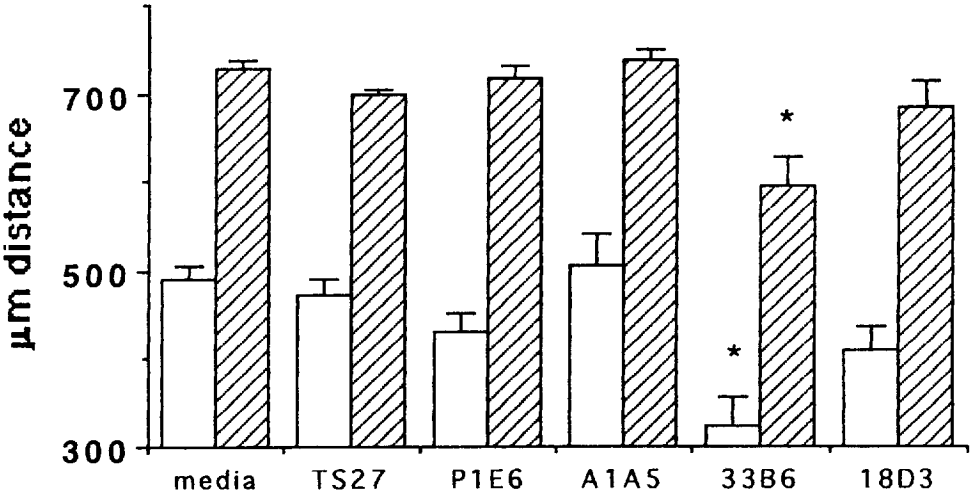
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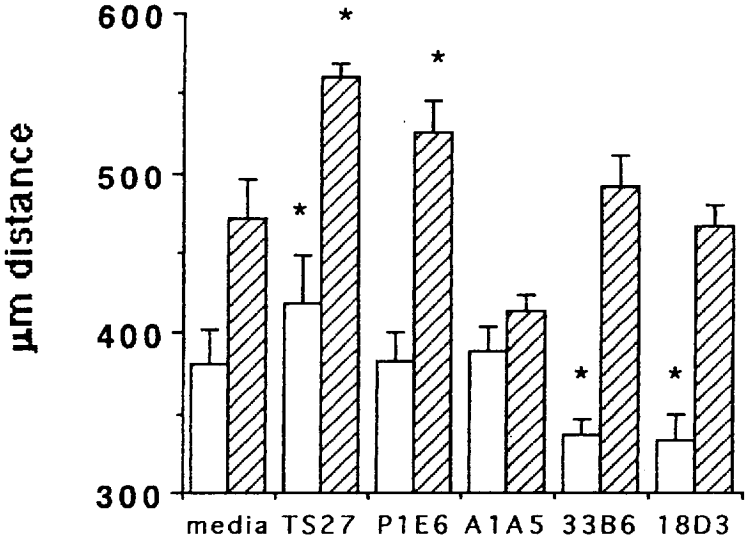
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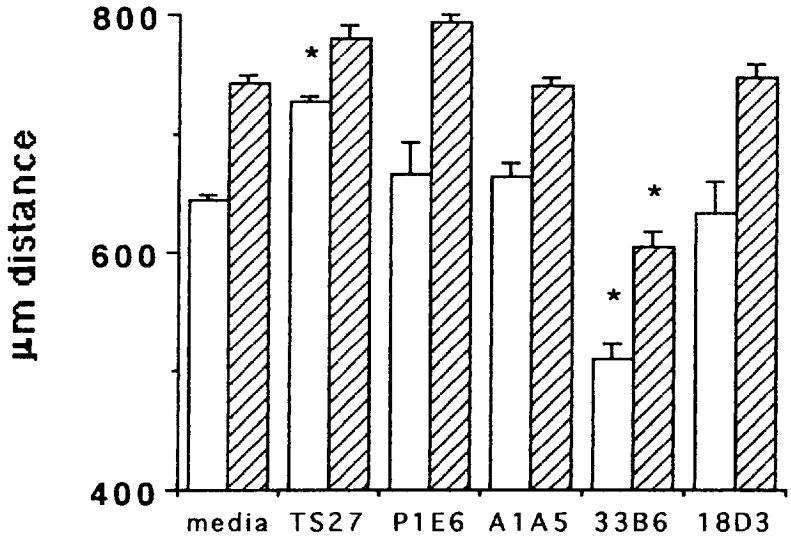
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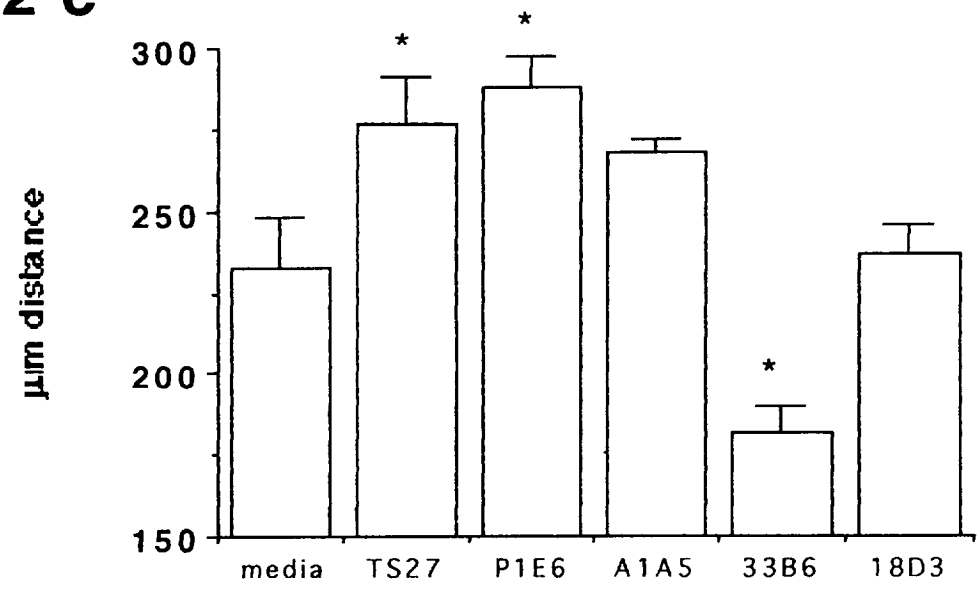
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µm distance
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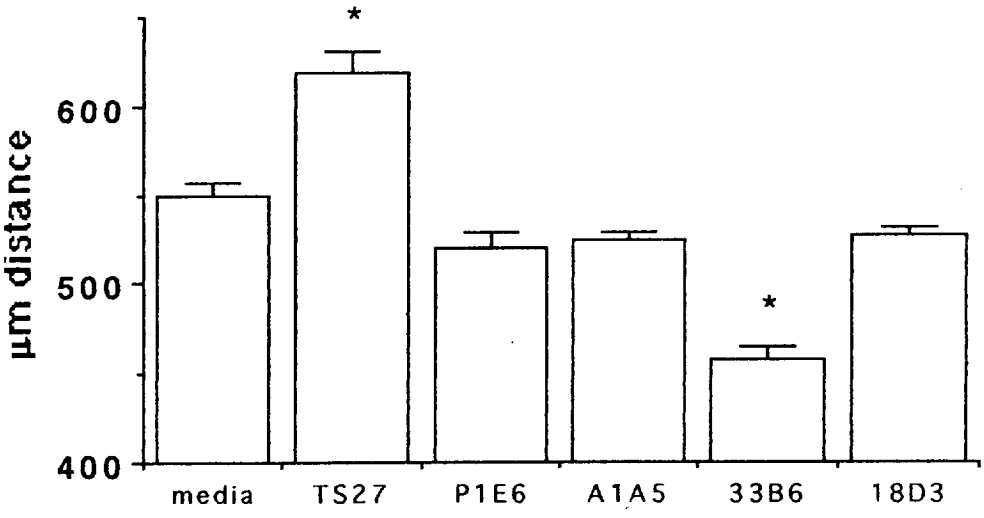
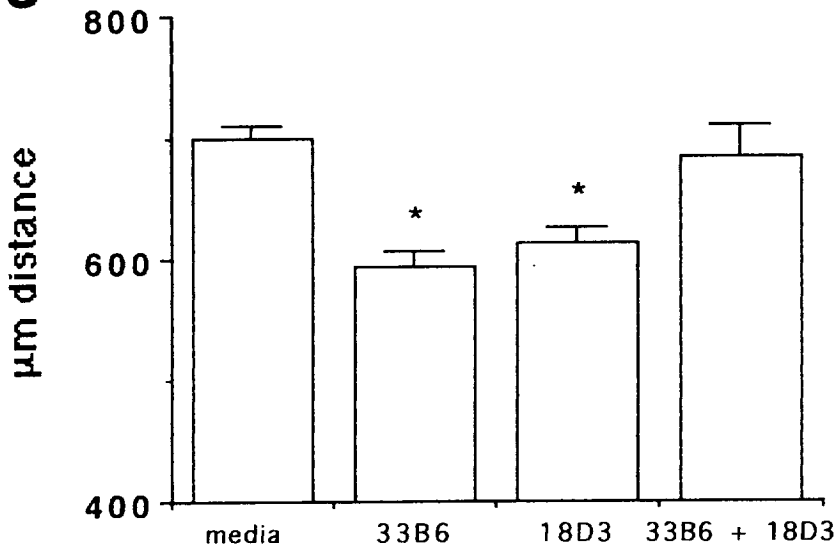
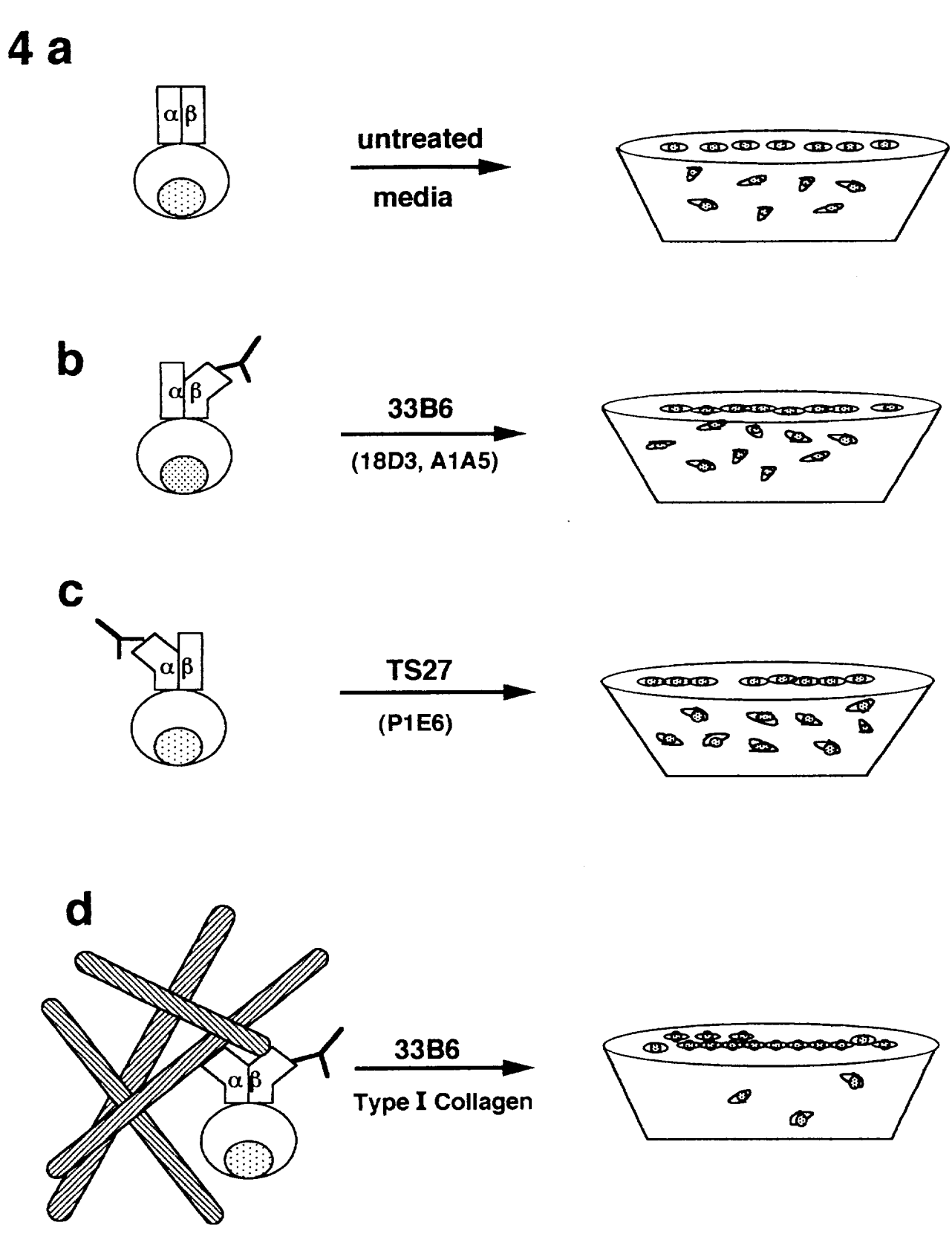
*
*
media
TS27
P1E6
A1A5
33B6
18D3

d


600
500
400
µm distance
*
*
media
TS27
P1E6
A1A5
33B6
18D3

3




4 a
α β
untreated
media
b
α β
33B6
(18D3, A1A5)
c
α β
TS27
(P1E6)
d
α β
33B6
Type I Collagen